PCT/CA2004/000006
06 February 2004 06.02.04

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Specification and Drawings, as originally filed, with Application for Patent Serial No: **2,414,487**, on January 13, 2003, by **FLORISYS INC., AND INSTITUTE FUR PFLANZENBIOCHEMIE**, assignee of Helmut Maucher, Otto Miersch, Claus Wasternack and Luc Varin, for "Methods and Genetic Sequences for Producing Male Sterile Plants, and Plants Genetically Modified to Alter Anther Development".

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(CIPO 68)
04-09-02

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ABSTRACT

The invention relates to methods and genetic sequences to produce male sterile plants. More particularly the present invention provides a genetic sequence encoding for a hydroxyjasmonic acid sulfotransferase and methods for producing transgenic plants using such a sequence. Furthermore, the present invention provides methods to rescue the male sterile phenotype observed in transgenic plants overexpressing the hydroxyjasmonate sulfotransferase and in plants deficient in jasmonic acid biosynthesis by the application of 12-hydroxyjasmonic acid.

METHODS AND GENETIC SEQUENCES FOR PRODUCING MALE STERILE PLANTS, AND PLANTS GENETICALLY MODIFIED TO ALTER ANTHER DEVELOPMENT

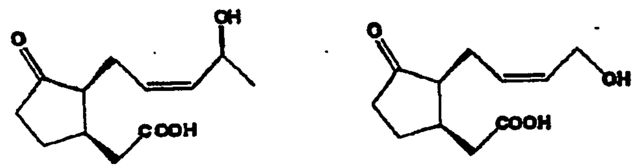
BACKGROUND OF THE INVENTION

a) Field of the invention

The invention relates to methods and genetic sequences to produce male sterile plants and to methods to rescue the male sterile phenotype.

b) Brief description of the prior art

There are several direct and indirect evidences for the involvement of jasmonic acid (JA) and related cyclopentanones, collectively called jasmonates in flower development (Wasternack and Hause (2002), *Prog Nucleic Acid Res Mol Biol* 72:165-221). For instance, *A. thaliana* mutants in JA biosynthesis or signaling are male sterile as a consequence of improper timing of anther and pollen development (Feys et al. (1994) *Plant Cell* 6: 751-759). A role in female reproductive development has also been attributed to jasmonates. For instance, the tomato *jai-1* mutant which is insensitive to the exogenous application of methyl jasmonate (MeJA) and cannot express defense-related genes in response to wounding was found to be female sterile (Li et al. (2001) *Plant Physiol.* 127:1414-1417). The species-specific difference in the requirement of jasmonates for the development of male or female gametophyte has yet to be explained. Finally, relatively high levels of jasmonates are found in the developing reproductive organs as compared to leaves and specific jasmonates such as JA tyramine conjugate and 12-hydroxyjasmonate (12-OHJA) are present in flowers (Miersch et al. (1998) *Phytochemistry* 47: 327-329).



11-hydroxyjasmonic acid 12-hydroxyjasmonic acid

Figure 1 Chemical structure of 11- and 12-hydroxyjasmonic acid

The inventors demonstrated that 11- and/or 12-OHJA (Figure 1) are required for proper anther development. First, they demonstrated that the exogenous application of 12-OHJA to the inflorescence of the *A. thaliana opr3* mutant deficient in JA biosynthesis rescued the male sterile phenotype. Furthermore, they demonstrated that the overexpression of the *A. thaliana AtST2a* gene encoding a 11-/12-OHJA sulfotransferase in transgenic tobacco led to a male sterile phenotype that could be rescued by the exogenous application of JA or 12-OHJA.

Many functions have been associated with jasmonate metabolites such as 12-hydroxyjasmonic acid and/or 11-hydroxyjasmonic acid. For instance, U.S. patent No 5,935,809, suggests the use of jasmonate for inducing plant defense mechanisms. U.S. patent No 5,814,581 describes a plant growth promoter composition comprising jasmonate and brassinolide as active ingredients and Tazaki (Japanese kokai 292220 (A) published April 3 1990, and patent application no 63-242432, filed September 29, 1988); Yoshihara *et al.* (1989), *Agric. Biol. Chem.* 53: 2835-2837, Matsuki *et al.* (1992), Biosci. Biotech. Biochem. 56: 1329.; and Koda and Okazawa (1988), *Plant Cell Physiol.* 29: 969), suggest the use of 12-hydroxyjasmonic acid for inducing tuber formation in potatoes. None of these documents disclose nor suggest that it is possible to produce male sterile plants by increasing in-vivo sulfonation of hydroxyjasmonates or by decreasing the synthesis of 11- and/or 12-OHJA.

Accordingly, there is a need for effective methods to produce male sterile plants that can be applied to all flowering plants and for methods to rescue the male sterile phenotype. There is also a need for plants genetically modified to be male sterile.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 shows the chemical structures of 11-hydroxyjasmonic acid and 12-hydroxyjasmonic acid.

Figure 2 shows the results of a Northern blot experiment with mRNA extracted from selected transgenic lines as compared to wild type.

Figure 3 is a composite picture showing the phenotype of flowers from transgenic *Nicotiana tobaccum* plants expressing the *AtST2a* gene under the control of a constitutive promoter (CaMV35S) compared to flowers from wild type non-transgenic plants (WT).

Figure 4 shows the results of the quantification of jasmonates in dissected tissues from wild type and mutant flowers.

Figure 5 shows the results of a normalization experiment. Treatments of the apex of transgenic plants for 14 days with 12-OHJA led to the rescue of the mutant phenotype and to the production of viable pollen

Figure 6: Shows nucleotide sequence of *AtST2a* gene (SEQ ID NO 1) taken from the GeneBank database (accession number NM_120783)

Figure 7: Shows the deduced amino acid sequence (SEQ ID NO 3) of the protein encoded by the *AtST2a* gene shown in Fig. 6.

Figure 8: Shows the nucleotide sequence of *AtST2b* gene (SEQ ID NO 2) taken from the GeneBank database (accession number NM_120782)

Figure 9: Shows the deduced amino acid sequence (SEQ ID NO 4) of the protein encoded by the *AtST2b* gene shown in Fig. 8.

DETAILED DESCRIPTION OF THE INVENTION

A) Definitions

In order to provide an even clearer and more consistent understanding of the specification, including the scope given herein to such terms, the following definitions are provided:

11-hydroxyjasmonic acid: 3-Oxo-2-(4-hydroxy-2-pentenyl)-cyclopentane-1-acetic acid. Its chemical structure is shown in Fig. 1.

11-hydroxyjasmonic acid sulfate: 3-Oxo-2-(4-hydroxysulfonyloxy-2-pentenyl)-cyclopentane-1-acetic acid

12-hydroxyjasmonic acid: 3-Oxo-2-(5-hydroxy-2-pentenyl)-cyclopentane-1-acetic acid. Its chemical structure is shown in Fig. 1.

12-hydroxyjasmonic acid sulfate: 3-Oxo-2-(5-hydroxysulfonyloxy-2-pentenyl)-cyclopentane-1-acetic acid.

Antisense: Refers to nucleic acids molecules capable of regulating the expression of a corresponding gene in a plant. An antisense molecule as used herein may also encompass a gene construct comprising a structural genomic gene, a cDNA gene or part thereof in reverse orientation relative to its or another promoter. Typically antisense nucleic acid sequences are not templates for protein synthesis but yet interact with complementary sequences in other molecules (such as a gene or RNA) thereby causing the function of those molecules to be affected.

Exogenous nucleic acid: A nucleic acid sequence (such as cDNA, cDNA fragments, genomic DNA fragments, antisense RNA, oligonucleotide) which is not normally part of a plant genome. The "exogenous nucleic acid" may be from any organism or purely synthetic. Typically, the "exogenous nucleic acid sequence" encodes a plant gene such as *AtST2a*, *AtST2b* or functional homologues of these genes.

Expression: The process whereby an exogenous nucleic acid, such as a nucleic acid sequence encoding a gene, is transcribed into a mRNA and afterwards translated into a peptide or a protein, in order to carry out its function, if any.

Functional homologue: Refers to a molecule having at least 50%, more preferably at least 55%, even more preferably at least 60%, still more preferably at

least 65-70%, and yet even more preferably greater than 85% similarity at the level of nucleotide or amino acid sequence to at least one or more regions of a given nucleotide or amino acid sequence. According to preferred embodiments of the present invention, the terms "functional homologue" refer to proteins or nucleic acid sequences encoding an enzyme having a substantially similar biological activity as 11- or 12-hydroxyjasmonate sulfotransferase and isoenzyme thereof. Such a functional homologue may exist naturally or may be obtained following a single or multiple amino acid substitutions, deletions and/or additions relative to the naturally occurring enzyme using methods and principles well known in the art. A functional homologue of a protein may or may not contain post-translational modifications such as covalently linked carbohydrate, if such modification is not necessary for the performance of a specific function. It should be noted, however, that nucleotide or amino acid sequences may have similarities below the above given percentages and still encode a 11- or 12-hydroxyjasmonate sulfotransferase-like molecule, and such molecules may still be considered within the scope of the present invention where they have regions of sequence conservation.

Genetic/nucleotide sequence: These terms are used herein in their most general sense and encompass any contiguous series of nucleotide bases encoding directly, or via a complementary series of bases, a sequence of amino acids comprising a hydroxyjasmonic acid sulfotransferase molecule, and more particularly a 11- or 12-OHJA sulfotransferase. Such a sequence of amino acids may constitute a full-length 11- or 12-OHJA sulfotransferase such as is set forth in SEQ ID No:1 and SEQ ID No:2 or an active truncated form thereof or a functional mutant, derivative, part, fragment, homologue or analogue thereof, or may correspond to a particular region such as an N-terminal, C-terminal or internal portion of the enzyme.

Genetic modification or genetic engineering: Refers to the introduction of an exogenous nucleic acid into one or more plant cells to create a genetically modified plant. Methods for genetically modifying a plant are well known in the art. In some cases, in may be preferable that the genetic modification is permanent such that the genetically modified plant may regenerate into whole, sexually

competent, viable genetically modified plants. A plant genetically modified in a permanent manner would preferably be capable of self-pollination or cross-pollination with other plants of the same species, so that the exogenous nucleic acid, carried in the germ line, may be inserted into or bred into agriculturally useful plant varieties.

Endogenous level(s): Refers to the amount of a given substance which is normally found in a plant (intrinsic) at a given time and stage of growth. Reference herein is made to the altering of the endogenous level of a compound or of an enzyme activity relating to an elevation or reduction in the compound's level or enzyme activity of up to 30% or more preferably of 30-50%, or even more preferably 50-75% or still more preferably 75% or greater above or below the normal endogenous or existing levels. The levels of a compound or the levels of activity of an enzyme can be assayed using known method and techniques.

Isolated nucleic acid molecule: Means a genetic sequence in a non-naturally-occurring condition. Generally, this means isolated away from its natural state or formed by procedures not necessarily encountered in its natural environment. More specifically, it includes nucleic acid molecules formed or maintained *in vitro*, including genomic DNA fragments, recombinant or synthetic molecules and nucleic acids in combination with heterologous nucleic acids such as heterologous nucleic acids fused or operably-linked to the genetic sequences of the present invention. The term "isolated nucleic acid molecule" also extends to the genomic DNA or cDNA or part thereof, encoding a hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-OHJA sulfotransferase, or a functional mutant, derivative, part, fragment, homologue or analogue of 11- or 12-OHJA sulfotransferase in reverse orientation relative to its or another promoter. It further extends to naturally-occurring sequences following at least a partial purification relative to other nucleic acid sequences. The term isolated nucleic acid molecule as used herein is understood to have the same meaning as nucleic acid isolate.

Plant: refers to a whole plant or a part of a plant comprising, for example, a cell of a plant, a tissue of a plant, an explant, or seeds of a plant. This term further contemplates a plant in the form of a suspension culture or a tissue culture

including, but not limited to, a culture of calli, protoplasts, embryos, organs, organelles, etc.

Similarity/Complementarity: In the context of nucleic acid sequences, these terms mean a hybridizable similarity under low, alternatively and preferably medium and alternatively and most preferably high stringency conditions, as defined below. Such a nucleic acid is useful, for example, in screening hydroxyjasmonic acid sulfotransferase genetic sequences, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase genetic sequences from various sources or for monitoring an introduced genetic sequence in a transgenic plant. The preferred oligonucleotide is directed to a conserved hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase genetic sequence or a sequence conserved within a plant genus, plant species and/or plant cultivar or variety.

Stringency: For the purpose of defining the level of stringency, reference can conveniently be made to Maniatis et al. (1982) at pages 387-389, and especially paragraph 11. A low stringency is defined herein as being in 4-6X SSC/1% (w/v) SDS at 37-45 °C for 2-3 hours. Depending on the source and concentration of nucleic acid involved in the hybridization, alternative conditions of stringency may be employed such as medium stringent conditions which are considered herein to be 1-4X SSC/0.5-1% (w/v) SDS at greater than or equal to 45°C for 2-3 hours or high stringent conditions considered herein to be 0.1-1X SSC/0.1-1.0% SDS at greater than or equal to 60° C. for 1-3 hours.

Transformed plant: Refers to introduction of an exogenous nucleic acid, typically a gene, into a whole plant or a part thereof, and expression of the exogenous nucleic acid in the plant.

Transgenic plant: Refers to a whole plant or a part thereof stably transformed with an exogenous nucleic acid introduced into the genome of an individual plant cell using genetic engineering methods.

Vector: A self-replicating RNA or DNA molecule which can be used to transfer an RNA or DNA segment from one organism to another. Vectors are particularly useful for manipulating genetic constructs and different vectors may have properties particularly appropriate to express protein(s) in a recipient during

cloning procedures and may comprise different selectable markers. Bacterial plasmids are commonly used vectors. Preferably, the vectors of the invention are capable of facilitating transfer of a nucleic acid into a plant cell and/or facilitating integration into a plant genome.

__B) General overview of the invention__

The present inventors have now discovered that it is possible to produce male sterile plants by increasing the activity of a hydroxyjasmonate sulfotransferase or by decreasing the activity of a jasmonic acid 11-/12-hydroxylase. Although many approaches may be used to achieve these effects, the approaches described hereinafter are preferably used according to the invention.

1) Overexpression of a hydroxyjasmonate sulfotransferase

An aspect of the invention contemplates a plant genetically modified to be male sterile when compared to a corresponding plant not genetically modified, wherein the genetically modified plant has a decreased endogenous level of at least one given compound of the jasmonate family selected preferably from the group consisting of 12-OHJA, glucoside of 12-OHJA, 12-hydroxymethyljasmonic acid, glucoside of 12-hydroxymethyljasmonic acid, 11-OHJA, glucoside of 11-OHJA, 11-hydroxymethyljasmonic acid, and glucoside of 11-hydroxymethyljasmonic acid as well as the amino acid conjugates of the above mentioned compounds, when compared to the corresponding non-genetically modified plant.

According to a preferred embodiment of the invention this is achieved by genetically modifying the plant so as to increase the expression of the sulfotransferase sulfonating 12-OHJA and/or 11-OHJA, or functional homologues of this sulfotransferase. More preferably, the plant is modified to increase the expression of at least one gene selected from the group consisting of *AtST2a*, *AtST2b* and functional homologues of *AtST2a* or of *AtST2b*.

SEQ ID NO 1 (Fig. 6 ; GeneBank: accession number NM_120783) corresponds to the gene *AtST2a* in *Arabidopsis thaliana*. SEQ ID NO 3 (Fig. 7) is an amino

acid sequence deduced from SEQ ID NO 1. This amino acid sequence is of public domain and can be retrieved from Genebank, accession number NM_120783. The *AtST2a* gene from *Arabidopsis thaliana* encodes a sulfotransferase that sulfonates 12-OHJA and 11-OHJA with high specificity. This hydroxyjasmonic acid sulfotransferase exhibits high affinity for its substrate with a Km value of 11 µM for 12-OHJA and 60 µM for 11-OHJA. The enzyme did not accept structurally related compounds such as cucurbic acid, arachidonyl alcohol or prostaglandins. Maximum enzyme activity was observed at pH 7.5 in Tris/HCl buffer and did not require divalent cations for activity.

SEQ ID NO 2 (Fig. 8; GeneBank: accession number NM_120782) corresponds to the gene *AtST2b* in *Arabidopsis thaliana*. SEQ ID NO 4 (Fig. 9) is an amino acid sequence deduced from SEQ ID NO 1. This amino acid sequence is of public domain and can be retrieved from Genebank, accession number NM_120782. Amino acid sequence alignment between SEQ ID NOS 3 and 4 indicates that they share 85% amino acid sequence identity and 92% similarity, suggesting that *AtST2a* and *AtST2b* encode isoenzymes.

The nucleic acid molecules contemplated herein may exist alone or in combination with a vector and preferably an expression-vector capable of facilitating transfer and expression of the nucleic acid into the plant cell and/or facilitating integration into the plant genome. Such a vector may, for example, be adapted for use in electroporation, microprojectile bombardment, Agrobacterium-mediated transfer or insertion via DNA or RNA viruses. The vector and/or the nucleic acid molecule contained therein may or may not need to be stably integrated into the plant genome. The vector may also replicate and/or express in prokaryotic cells. Preferably, the vector molecules or parts thereof are capable of integration into the plant genome. The nucleic acid molecule and/or the vector may additionally contain a promoter sequence capable of directing expression of the nucleic acid molecule in a plant cell. The nucleic acid molecule and/or the vector may also be introduced into the cell by any number of means such as those described above.

The present invention is exemplified using nucleic acid sequences derived from *Arabidopsis thaliana* since this plant is commonly studied in and it represents

a convenient and easily accessible source of material. However, one skilled in the art will immediately appreciate that similar sequences can be isolated from any number of sources such as other plants or certain microorganisms (e.g. fungi or bacteria). All such nucleic acid sequences encoding directly or indirectly a hydroxyjasmonic acid sulfotransferase are encompassed by the present invention regardless of their source. Examples of other suitable sources of genes encoding hydroxyjasmonic acid sulfotransferase include, but are not limited to Brassica napus, *Solanum tuberosum, Solanum demissum, Nicotiana tobaccum, Helianthus tuberosus and Astragalus complanatus*

According to a preferred embodiment, the method comprises the step of:

a) introducing into a cell of a suitable plant an exogenous nucleic acid molecule comprising a sequence of nucleotides encoding a plant hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase;
b) regenerating a transgenic plant from the cell; and where necessary
c) growing the transgenic plant for a time and under conditions sufficient to permit expression of the nucleic acid sequence into a plant hydroxyjasmonic acid sulfotransferase, preferably a 11- or 12-hydroxyjasmonic acid sulfotransferase.

The details of the construction of transgenic plants are known to those skilled in the art of plant genetic engineering and do not differ in kind from those practices which have previously been demonstrated to be effective in tobacco, petunia and other model plant species (e.g. electroporation, microprojectile bombardment, Agrobacterium-mediated transfer or insertion via DNA or RNA viruses). One skilled in the art will immediately recognize the variations applicable to the methods of the present invention, such as increasing the expression of the sulfotransferase naturally present in a target plant leading to the production of male sterile plants. The present invention, therefore, extends to all transgenic plants containing all or part of the nucleic acid sequence of the present invention, and/or any homologues or related forms thereof and in particular those transgenic plants which exhibit a male sterile phenotype.

2) Reduction of expression of a jasmonic acid 11-/12-hydroxylase

An aspect of the invention contemplates a plant genetically modified to be male sterile when compared to a corresponding plant not genetically modified, wherein the genetically modified plant has a decreased endogenous level of at least one given compound of the jasmonate family selected preferably from the group consisting of 12-OHJA, glucoside of 12-OHJA, 12-hydroxymethyljasmonic acid, glucoside of 12-hydroxymethyljasmonic acid, 11-OHJA, glucoside of 11-OHJA, 11-hydroxymethyljasmonic acid, and glucoside of 11-hydroxymethyljasmonic acid as well as the amino acid conjugates of the above mentioned compounds, when compared to the corresponding non-genetically modified plant.

According to a preferred embodiment of the invention this is achieved by genetically modifying the plant so as to decrease the expression of the hydroxylase(s) responsible for the conversion of jasmonic acid to 11- and/or 12-OHJA. Reduction in the jasmonic acid 11- and/or 12-hydroxylase expression or activity can be achieved by antisense technology, by knockout of the gene, by expression of an antibody inhibiting the hydroxylase activity or by the expression of a ribozyme specific for the mRNA of the jasmonic acid to 11- and/or 12-hydroxylase.

3) Inhibition of the activity of a jasmonic acid 11-/12-hydroxylase

In accordance with the present invention, the male sterile phenotype can be obtained by the application of an inhibitor of the jasmonic acid 11- and/or 12-hydroxylase. The inhibitor can be part of a composition for producing male sterile plants. The carrier or diluent can be a solvent such as water, oil or alcohol. The composition may also comprise other active agents such as fertilizers and growth regulators. The inducing composition may also be formulated with emulsifying agents in the presence or absence of fungicides or insecticides, if required. The precise amount of compound employed in the practice of the present invention will depend upon the type of response desired, the formulation used and the type of plant treated.

3) Complementation of the male sterile phenotype

In accordance with the present invention, the male sterile phenotype of the plants overexpressing the hydroxy-jasmonate sulfotransferase was rescued by the application of a solution of jasmonic acid or 12-OHJA. Structural analogs of the above mentioned compounds could also be used to rescue the phenotype. Alternatively, the male sterile phenotype could be rescued by the application of an inhibitor of the 11-/12-OHJA sulfotransferase.

The above mentioned compounds, can be part of a composition for recovering male fertility. The carrier or diluent can be a solvent such as water, oil or alcohol. The composition may also comprise other active agents such as fertilizers and growth regulators. The inducing composition may also be formulated with emulsifying agents in the presence or absence of fungicides or insecticides, if required. The precise amount of compound employed in the practice of the present invention will depend upon the type of response desired, the formulation used and the type of plant treated.

The inventors also demonstrated that the male sterile phenotype of jasmonic acid deficient mutant plants could be rescued by the application of 12-OHJA.

EXAMPLES

The following examples are illustrative of the wide range of applicability of the present invention. The invention is not restricted to the production of male sterile tobacco plants but can be applied to all flowering plant species. It should readily occur that the recognition of producing male sterile plants according to methods of the present invention in connection with other plants not specifically illustrated herein, is readily within the capabilities of one skilled in the art. The following examples are intended only to illustrate the invention and is not intended to limit its scope. Modifications and variations can be made therein without departing from the spirit and scope of the invention.

The following experimental procedures and materials were used for the examples set fort below.

A) MATERIAL AND METHODS

Studies using a vector:

For transgenic studies a EcoR1-HindIII cassette, from the plasmid pBI-525 comprising two CaMV 35S promoters in tandem followed by an AMV translational enhancer and a NOS terminator, was ligated to the plasmid pBI-101 which was previously digested with the same restriction endonucleases. The resulting vector called pBI-101-525 contained two CaMV 35S minimal promoters in tandem followed by an AMV translational enhancer, a NOS terminator and a kanamycin resistance gene. *AtST2a* cDNA (SEQ ID NO 1; Fig. 7) was cloned in the sense orientation at the BaMHI site in a polylinker lying downstream of the AMV enhancer. Various other promoters may be used to drive the expression of an exogenous gene in a plant. For example the ubiquitin promoter may be used for constitutive expression. Alternatively, inducible promoters may also be used such as the ethanol-inducible promoter or the glucocorticoid-inducible promoter.

Agrobacterium transformation:

A. tumefaciens strain LBA4404 was transformed with the *AtST2a*-pBI-101-525 sense construct by the method described in Gynheung *et al.* (1988) Biology Manual, A3:1-19.

Nicotiana tobaccum transformation:

Transgenic tobacco plants were produced using the leaf disk transformation method described by Horsch, R.B. et al. (1984) Science, Vol. 227, 1229-1231.

Northern blot of mRNA extracts

Total RNA was extracted from frozen tissues by the use of phenol/chloroform/isoamyl alcohol 25:24:1 according to the method described by Sambrook et al (1989). 20 µg of total RNA per lane was subjected to electrophoresis and Northern blot analysis was performed according to Sambrook et al. (1989). Blots were hybridized at 65°C for 16 h with a ^{32}P-labelled fragment of *A. thaliana AtST2a* cDNA encompassing the entire coding sequence.

Western blot analysis

For the analysis of *AtST2a* expression, leaves from T1 and T2 plants were ground in liquid nitrogen, and the powder was boiled in 2X SDS sample buffer. Protein extracts were separated by SDS-polyacryamide gel electrophoresis on 12% polyacrylamide gels and transferred onto nitrocellulose membrane. AtST2a was immunodetected using anti-AtST2a polyclonal antibodies (dilution 1:1000) and goat anti-rabbit secondary antibodies conjugated with alkaline phosphatase (dilution 1:3000 ; Bio Rad). To confirm equal loading of each sample, protein extracts were run on SDS-PAGE and stained with Coomassie blue.

Plant growth conditions

Wild type (SNN) and transgenic plants were grown in soil under greenhouse conditions with a 16 hours photoperiod.

Normalization experiments

The apex of T2 plants of line 7 was soaked daily in a solution containing either: 0.05% Tween20 in water (control), 0.05% Tween20 in water containing 50 µM MeJA or 0.05% Tween20 in water containing 100 µM 12-OHJA. The treatments were started approximately 7 days before the appearance of the first flower buds and lasted a total of 14 days. A minimum of 5 plants for each treatment were analyzed in this study.

B) RESULTS

The inventors demonstrated that it is possible to generate male sterile transgenic tobacco plants by heterologous expression of the *A. thaliana* gene *AtST2a* encoding the 12-OHJA sulfotransferase. The *AtST2a* gene was introduced in *Nicotiana tobaccum* plants by Agrobacterium-mediated transformation. Plants were regenerated and transformed plants were selected by resistance to kanamycin. Transformation was confirmed by Southern, Northern and Western blot. 29 independent transgenic lines were generated. Figure 2 shows a photograph of a Northern blot for three selected transgenic lines. Plants from four lines (including line 7) exhibited a male sterile phenotype and the phenotype was

found to correlate with a high level of *AtST2a* expression (Figure 3 and 2C). Except for the male sterile phenotype, no other phenotypic alterations were observed in the transgenic lines.

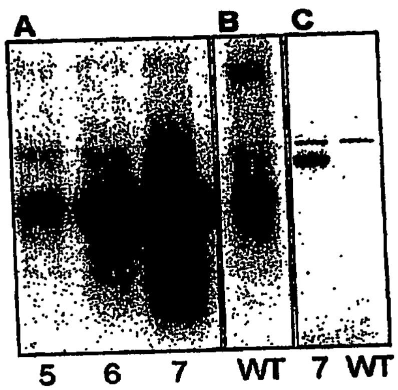


Figure 2 A) Northern blot of three selected transgenic lines hybridized with a ^{32}P-labelled *AtST2a* probe. 5, 6 and 7 indicate independent transgenic lines. WT indicates wild type plants B) Northern blot of wild type RNA hybridized with a ^{32}P-labelled *AtST2a* probe. C) Western blot of protein extracts from Line 7 and from wild type plants. The intense band in the line 7 sample corresponds to the expected molecular mass of the recombinant AtST2a protein.

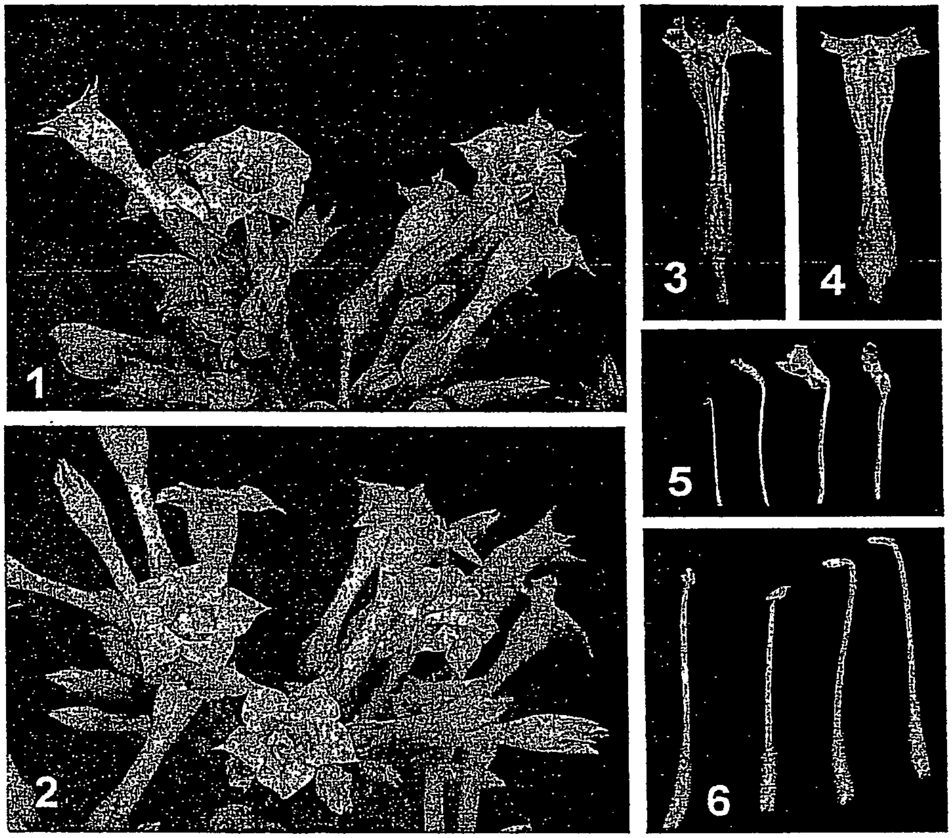


Figure 3 Phenotypic analysis of the *AtST2a* expression line 7 as compared with wild type. 1) Wild-type SNN inflorescence. 2) Transgenic line 7 inflorescence. 3) Dissected flower from line 7. 4) Dissected flower from wild type SNN. 5) Dissected anthers from line 7 flower. 6) Dissected anthers from wild type SNN flower

These results clearly indicate that it is possible to produce male sterile plants by altering the level of the enzyme that sulfonates 12-OHJA. It is also predicted that inhibition of the expression of the endogenous jasmonic acid 11-/12-hydroxylase will produce the same effect.

The level of different jasmonates were quantified in dissected flower tissues of the transgenic line 7 as compared with wild type. The results presented in Figure 4 show that anthers are the preferential site of accumulation of 12-OHJA in wild type flowers. In contrast, a drastic reduction in the amount of 12-OHJA in the anthers of the transgenic plant was observed as compared with wild type. These results

confirm that the overexpression of the 12-OHJA sulfotransferase lead to a decrease of 12-OHJA in the anthers of transgenic tobacco plants.

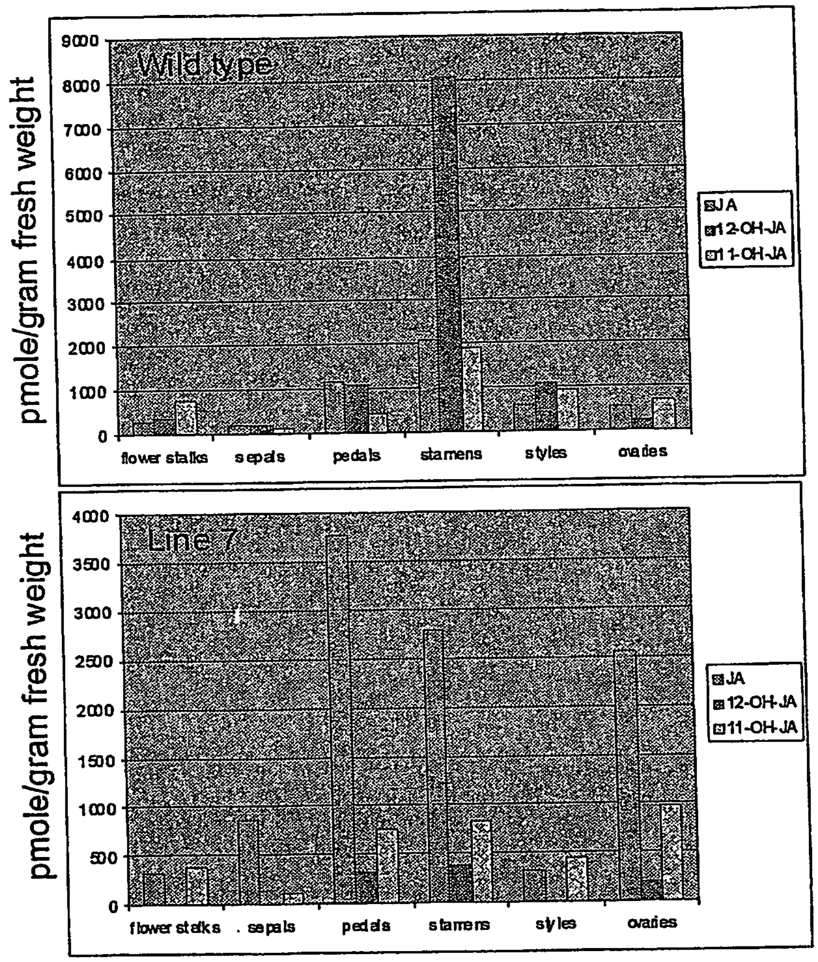


Figure 4. Quantification of jasmonates in dissected flower tissues from wild type and transgenic line 7.

In order to rescue the phenotype, the apex of transgenic plants (line 7) were soaked daily for 2 minutes in a solution containing 100 µM 12-OHJA dissolved in aqueous 0.05% TWEEN20. The application was started approximately 7 days prior to the appearance of the first flower bud and was continued for 7 days after the first flower appeared. The results show that the application of 12-OHJA

restored normal anther development (Figure 5). Similar results were obtained with 50 µM MeJA which is a precursor of 12-OHJA biosynthesis (data not shown). Control treatments with the carrier solution did not restore normal anther development. Application of 12-OHJA was also shown to rescue the male sterile phenotype of the *A. thaliana opr3* mutant (data not shown).

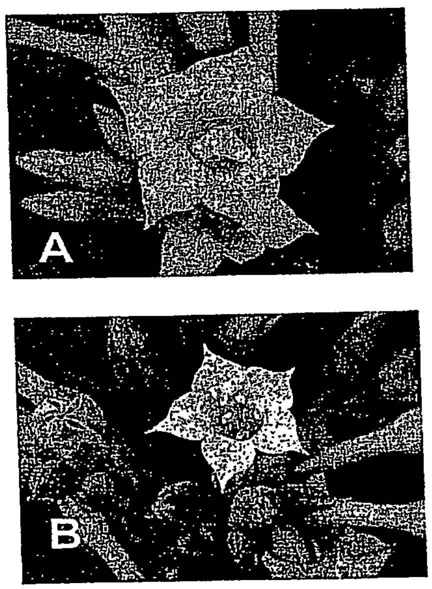


Figure 5. Results of a normalization experiment A) Flower from a line 7 plant treated with 0.05% Tween20 in water. B) Flower from a line 7 plant treated with 0.05% Tween20 in water containing 100 µM 12-OHJA

These results demonstrate that the male sterile phenotype associated with a deficiency in 12-OHJA observed in the tobacco transgenic line 7 and in the *A. thaliana opr3* mutant can be rescued by the application of 12-OHJA.

GeneBank accession number: NM_120783
SEQ ID NO 1

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   1 accaacacac aaagattcca ttacaaataa acaattttca tatatatcta taacaaaaaa
  61 aaacaatggc tacctcaagc atgaagagca ttccaatggc gatcccaagt ttctccatgt
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 181 atgaagggct aagctgcgag ttccaagaga tgttggattc tcttcctaag gagagaggat
 241 ggagaactcg ttacctttac ctattccaag ggttttggtg ccaagccaaa gagattcaag
 301 ccatcatgtc tttccaaaaa catttccaat ccctcgaaaa cgacgtcgtt ctcgccacca
 361 tacctaaatc cggtacaacc tggctaaaag ctttaacttt caccatcctt aaccgtcacc
 421 ggtttgatcc ggttgcctcg agtaccaacc accctctttt cacttccaac cctcatgacc
 481 ttgtaccttt cttcgagtac aagctttacg ccaacggaga tgttcccgat ctctcgggtc
 541 tagccagtcc aagaacgttc gcaacccact taccgttcgg ttccctaaag gaaacgatcg
 601 agaaacccgg tgtgaaggtc gtgtacttgt gccggaaccc gtttgacaca ttcatctctt
 661 cgtggcatta caccaacaac atcaaatccg agtcagtgag cccagtcttg ctagaccaag
 721 cttttgatct gtattgccgg ggagtgatcg ggtttggccc gttttgggaa cacatgttgg
 781 gatactggag agagagcttg aagagaccag agaaagtctt ctttttaagg tacgaggatc
 841 tcaaagacga catcgagacc aacttgaaga ggcttgcaac tttcttagag cttcctttca
 901 ccgaagaaga ggaacgaaag ggagttgtga aggctatcgc cgagctgtgt agcttcgaga
 961 atctgaagaa gttggaggtg aacaagtcaa acaagtcgat caagaacttt gagaatcgat
1021 tcttgtttcg gaaaggagaa gtgagtgatt gggttaacta tttgtcacct tcacaagtgg
1081 aaagattgtc agccttagtg gatgacaagt taggtggatc tggtctcact ttcaggttga
1141 gctaaatata aggccacgtg cccccatttc tactcttgtt ctgagggcct actatatacg
1201 ttaagctaag ttaaggcagt tgtattgttg ttacagatag acatcgaagc aacgtaacgt
1261 ccataattaa gtt
```

Figure 6. Nucleotide sequence of *AtST2a*

GeneBank accession number: NM_120783
SEQ ID NO 3

```
                    MATSSMKSIPMAIPSFSMCHKLELLKEGKTRDVPKAEEDEGLSC
EFQEMLDSLPKERGWRTRYLYLFQGFWCQAKEIQAIMSFQKHFQSLENDVVLATIPKS
GTTWLKALTFTILNRHRFDPVASSTNHPLFTSNPHDLVPFFEYKLYANGDVPDLSGLA
SPRTFATHLPFGSLKETIEKPGVKVVYLCRNPFDTFISSWHYTNNIKSESVSPVLLDQ
AFDLYCRGVIGFGPFWEHMLGYWRESLKRPEKVFFLRYEDLKDDIETNLKRLATFLEL
PFTEEEERKGVVKAIAELCSFENLKKLEVNKSNKSIKNFENRFLFRKGEVSDWVNYLS
PSQVERLSALVDDKLGGSGLTFRLS
```

Figure 7. Deduced amino acid sequence of *AtST2a*

GeneBank accession number NM_120782
SEQ ID NO 2

```
   1 atgtgtcaca agcccgagct ccttaaggaa ggcaaaagcg aaggccaaga agaagaaggg
  61 ctaagctacg agttccaaga gatgttggac tctcttccta aggagagagg acggagaaat
 121 cgttaccttt acttattcca agggtttcgg tgccaagcta aggagattca agctatcacg
 181 tctttccaaa aacattttca gtcccttcca gacgacgttg tcctcgccac catacctaaa
 241 tctggcacaa cctggttaaa agctttaact ttcaccatcc ttacccgtca tcggtttgat
 301 ccggtttcct catcaagttc cgaccacccct cttctcacat ccaaccctca cgacctcgta
 361 cctttcttcg agtacaagct ttacgccaac ggaaatgttc ccgatctctc gggtctagcc
 421 agtccaagaa cattcgcaac ccacgtaccg ttcggtgccc ttaaggattc ggtcgagaat
 481 cccagtgtga aggttgtgta cctgtgccgg aacccgtttg acacattcat ctccatgtgg
 541 cattacatca acaacatcac ttccgagtca gtgagcgcag tcttgctaga cgaagctttt
 601 gatctatatt gccggggatt actgatcgga tttggcccgt tttgggaaca catgttggga
 661 tactggagag agagcttgaa gaggccagag aaagtcttat ttttaaagta cgaggatctc
 721 aaagaagaca tcgagaccaa cttgaagaag ctagcaagtt tcttaggact tcctttcacc
 781 gaagaagagg aacaaaaggg agttgtgaaa gctatcgctg atctgtgtag ctttgagaat
 841 ctgaagaagt tggaggtgaa caagtcaagc aaattgatcc agaactatga gaaccggttc
 901 ttgtttagga aaggagaagt gagtgatttg gttaactatt tgtcgccatc acaagtggaa
 961 agattgtcag ccttagtgga tgacaagtta gctggatctg gtctcacttt cagattgagt
1021 taa
```

Figure 8. Nucleotide sequence of *AtST2b*

GeneBank accession number NM_120782
SEQ ID NO 4

```
                MCHKPELLKEGKSEGQEEEGLSYEFQEMLDSLPKERGRRNRYLY
LFQGFRCQAKEIQAITSFQKHFQSLPDDVVLATIPKSGTTWLKALTFTILTRHRFDPV
SSSSSDHPLLTSNPHDLVPFFEYKLYANGNVPDLSGLASPRTFATHVPFGALKDSVEN
PSVKVVYLCRNPFDTFISMWHYINNITSESVSAVLLDEAFDLYCRGLLIGFGPFWEHM
LGYWRESLKRPEKVLFLKYEDLKEDIETNLKKLASFLGLPFTEEEEQKGVVKAIADLC
SFENLKKLEVNKSSKLIQNYENRFLFRKGEVSDLVNYLSPSQVERLSALVDDKLAGSG
LTFRLS
```

Figure 9. Deduced amino acid sequence of *AtST2b*